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Molecular Cloning and Characterization of *acrA* and *acrE* Genes of *Escherichia coli*

DZWOKAI MA,[1,2]* DAVID N. COOK,[1,2]† MARIE ALBERTI,[2] NING G. PON,[2] HIROSHI NIKAIDO,[3] AND JOHN E. HEARST[1,2]

Department of Chemistry[1] and Department of Molecular and Cell Biology,[3] University of California, and Division of Structural Biology, Lawrence Berkeley Laboratory,[2] Berkeley, California 94720

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The DNA fragment containing the *acrA* locus of the *Escherichia coli* chromosome has been cloned by using a complementation test. The nucleotide sequence indicates the presence of two open reading frames (ORFs). Sequence analysis suggests that the first ORF encodes a 397-residue lipoprotein with a 24-amino-acid signal peptide at its N terminus. One inactive allele of *acrA* from strain N43 was shown to contain an IS2 element inserted into this ORF. Therefore, this ORF was designated *acrA*. The second downstream ORF is predicted to encode a transmembrane protein of 1,049 amino acids and is named *acrE*. Genes *acrA* and *acrE* are probably located on the same operon, and both of their products are likely to affect drug susceptibilities observed in wild-type cells. The cellular localizations of these polypeptides have been analyzed by making *acrA*::Tn*phoA* and *acrE*::Tn*phoA* fusion proteins. Interestingly, AcrA and AcrE share 65 and 77% amino acid identity with two other *E. coli* polypeptides, EnvC and EnvD, respectively. Drug susceptibilities in one *acrA* mutant (N43) and one *envCD* mutant (PM61) have been determined and compared. Finally, the possible functions of these proteins are discussed.

The global level of DNA supercoiling in *Escherichia coli* is controlled by a competition between two complementary enzymes, topoisomerase I and DNA gyrase. The division of labor between topoisomerase I and gyrase is evident at the biochemical level, since topoisomerase I removes only negative supercoils under physiological conditions (14) whereas gyrase can relax positive supercoils or introduce negative supercoils (8). It appears that topoisomerase I and gyrase compoise a homeostatic system for maintaining DNA supercoiling near an optimal set point in vivo (4, 24).

Some of the earliest evidence that topoisomerase I and gyrase work together as a homeostatic system came from genetic studies on the effects of deletions of the gene for topoisomerase I in *E. coli* (5). Deletion of *topA* is deleterious to *E. coli*, and Δ*topA* mutants readily acquire compensatory mutations at other loci. A number of the compensatory mutations were mapped to *gyrA* or *gyrB*, and they reduced the level of gyrase activity in the cell (5, 37). Not all compensatory mutations mapped to the genes for gyrase, however. One class of compensatory mutations occurred near *tolC* at 66 min on the genetic map (38).

Mutations at the *acrA* locus also occurred in response to deletion of *topA* (5). These mutations were not truly compensatory, since by themselves they did not restore viability to Δ*topA* transductants (5). However, under specific growth conditions, others have found that *acrA* lesions are compensatory for deletion of *topA*. Dorman et al. (6) showed that Δ*topA* could be transduced directly into an *acrA* mutant background when cells are grown in a low-osmolarity medium. Nothing is known about why *acrA* mutations follow deletion of *topA* or how these *acrA* mutants permit loss of *topA* during growth in a low-osmolarity medium.

Gene *acrA*, which maps between *proC* and *purE* at 10.5 min on the *E. coli* chromosome, was first identified because mutations at this locus led to hypersusceptibility to acridines used in curing the F factor from *E. coli* (27, 29, 31). Subsequent studies showed that mutations at the same locus also determined susceptibility to other basic dyes, detergents, and certain antibiotics (3, 28). Thus, *acrA* was originally thought to contribute to the integrity of *E. coli* membranes. Nakamura and Suganuma (30) first suggested that *acrA* mutations affected the biosynthesis of a 58-kDa inner membrane protein and that more acriflavine was bound to the inner membrane in an *acrA* mutant. However, this model does not easily explain the hypersusceptibility of *acrA* mutants toward a broad spectrum of other drugs. It is also not consistent with the hypothesis that it is the outer membrane, not the inner membrane, that constitutes the major barrier for most drugs (35). Coleman and Leive (3) did not detect any change in composition of inner or outer membrane proteins in an *acrA* mutant. Instead, they observed a 50% reduction in the phosphate content of the lipopolysaccharides, but these data were later retracted (18). To date, no defects in membrane structure have been unambiguously shown to be associated with mutations of *acrA*, and the basis for drug hypersusceptibility in *acrA* mutants remains an enigma.

Here we report the molecular cloning of genes at the *acrA* locus by complementation of the drug hypersusceptibility in one *acrA* mutant, N43. Sequence analysis identified two open reading frames (ORFs) at the previously assigned *acrA* locus. In N43, an IS2 insertion was found in the upstream ORF, which has been designated *acrA*. The downstream ORF has been designated *acrE*. Hydropathy plots and Tn*phoA* fusions have been used to analyze the topologies of AcrA and AcrE. Moreover, AcrA and AcrE are highly homologous at the amino acid level to EnvC and EnvD, respectively. The effects of mutations on drug susceptibility at these loci have also been compared. Finally, the potential functions of these proteins and the possible mechanisms by which *acrA* mutations alleviate the deleterious effects of *topA* deletion are discussed.

* Corresponding author.
† Present address: Steritech Inc., Concord, CA 94520.

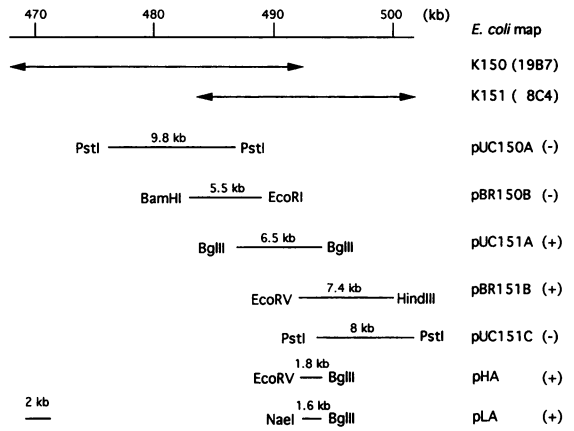

FIG. 1. Localization and cloning of the *acrA* and *acrE* genes by drug susceptibility complementation. Five different restriction fragments from two Kohara clones (K150 and K151) were inserted into either pUC19 or pBR322 (see Material and Methods). The size of each fragment was estimated by comparing its mobility against the fragments of the 1-kb DNA ladder (from GIBCO BRL) in a 1% agarose gel. The regions on the *E. coli* physical map which are contained by K150 and K151 are taken from Miller (26) and Rudd et al. (39). (+) and (−) represent the results of the drug sensitivity complementation.

MATERIALS AND METHODS

Bacteria strains and growth conditions. Strains used for cloning the *acrA* locus and determining drug susceptibility due to mutation at the *acrAE* loci were W4573 (F⁻ K-12 *lac ara mal xyl mtl gal rpsL*) and its isogenic *acrA* mutant derivative N43 (W4573 *acrA1*). Drug susceptibility due to mutation at the *envCD* loci was assayed by using P678S^R [*thr-1 ara-13 leuB6 tonA2 lacY1 supE44? gal-6* λ⁻ *rpsL135 malA1*(λ^r) *xyl-7 thi-1 mtl-2*] and PM61 [*thr-1 ara-13 leuB6 tonA2 lacY1 supE44 gal-6* λ⁻ *rpsL135 malA1*(λ^r) *xyl-7 thi-1 envC61*]. Tn*phoA* fusion constructs were assayed in strain CC118 [*araD139* Δ(*ara, leu*)*7697* Δ*lacX74 phoA*Δ*20 galE galK thi rpsE rpoB arg*E(Am) *recA1*]. LB medium (10 g of Bacto Tryptone, 5 g of Bacto Yeast Extract, and 10 g of NaCl per liter, pH 7) was used to grow all bacterial cultures unless specifically indicated. The antibiotics streptomycin (100 μg/ml), ampicillin (35 μg/ml for N43 and 100 μg/ml for other strains), and kanamycin (35 μg/ml) were used for selection.

Plasmids and phages. Kohara λ phage clones K150, K151, and K530 were kindly provided by Fred Blattner (University of Wisconsin). K150 and K151 span the 10.5-min region of the *E. coli* chromosome (17, 26, 39), and K530 has been previously mapped by hybridization to contain *envCD* (15). To isolate *acrA*, K150 and K151 were subcloned into either pUC19 or pBR322 to generate plasmids pUCK150A, pBRK150B, pUCK151A, pBRK151B, and pUCK151C (Fig. 1). Plasmids pUCK150A and pBRK150B contained fragments from K150. Plasmids pUCK151A, pBRK151B, and pUCK151C contained fragments from K151. Plasmid pHA contained a 1.8-kb *Bgl*II-to-*Eco*RV fragment from K151 cloned into pUC19, and pLA contained a 1.6-kb *Bgl*II-to-*Nae*I fragment covering most of the same region from K151 cloned into pACYC177.

Complementation analysis. The inhibitory concentrations for growth in the presence of novobiocin and mitomycin C were determined on LB plates for strains W4573 and N43. For W4573, no growth inhibition was observed at 30 μg/ml for novobiocin and 1 μg/ml for mitomycin C. For N43, inhibition occurred at 5 and 0.1 μg/ml, respectively. Plasmids containing subclones of K150 and K151 were transformed into N43 and selected for ampicillin resistance. Transformants were then restreaked onto plates containing either 30 μg of novobiocin per ml or 0.3 μg of mitomycin C per ml. Complementation was scored after overnight growth on plates at 37°C.

DNA sequencing. Sequencing was performed by using the nucleotide kit for sequencing with Sequenase T7 DNA polymerase and 7-deaza-dGTP (catalog no. 70750; United States Biochemical).

PCR amplifications. Genomic DNA from N43 was purified as described by Sambrook et al. (40). The polymerase chain reaction (PCR) was performed in a total volume of 100 μl containing 3 ng of genomic DNA, 1 μM forward and reverse primers, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 10% glycerol, 200 μM (each) of the four deoxynucleoside triphosphates, and 5 U of *Taq* DNA polymerase (Perkin Elmer-Cetus). The reactions were carried out for 30 cycles with the following temperature profile: 94°C for 1 min, 45°C for 1 min, and 72°C for 2 min.

Construction of Tn*phoA* fusions. λTn*phoA*-1 was used for the purpose of inserting Tn*phoA* into multicopy plasmid pHA. All other procedures were as described by Manoil and Beckwith (22).

Alkaline phosphatase activity assays. Alkaline phosphatase activity was assayed according to a standard protocol. A 1-ml aliquot of cells was removed from the culture at an optical density at 600 nm (OD_{600}) of 0.45 to 0.55, immediately frozen in liquid nitrogen, and stored at −70°C. To assay samples, cells were thawed at 15°C and transferred to ice upon melting. A 50-μl sample was mixed with 950 μl of ice-cold 1 M Tris-HCl, pH 8.0. Cells were permeabilized with 50 μl of toluene and allowed to equilibrate at 37°C for 5 min before the assay. A 200-μl aliquot of Sigma 104-105 reagent (*p*-nitrophenyl phosphate) was added and mixed by inversion. The samples were then incubated for 14 min. Reactions were quenched by adding 100 μl of 1 M K_2HPO_4. After cell debris was spun down, the A_{420} was recorded and corrected for residual scattering at 550 nm. Cell scattering on the original samples at 600 nm was also measured, and phosphatase activity was calculated from the following formula: PhoA activity = $1{,}000 \times [A_{420} - 1.75 \times (A_{550})]/[(A_{600}) \times T \times V]$, where T is the time in minutes and V is the volume of the cell culture in milliliters.

Drug inhibition measurements. The MICs of various antibiotics were determined in N43 and PM61 and their isogenic parental strains, W4573 and P678S^R. Bacteria were grown overnight at 37°C in LB with 100 μg of streptomycin per ml. A 1:100 dilution was made into prewarmed (37°C) LB. The cultures were then removed from 37°C after reaching an OD_{600} of 0.4, cooled to room temperature, and stored at 4°C. The cultures were used for determination of MIC within 6 h. To measure the MIC, the stored cultures were inoculated at a density of 5×10^4 cells per ml into LB in the presence of twofold increasing concentrations of the drug under investigation. Cell growth, measured by OD_{600}, was assayed after an 18-h incubation on a shaker (150 rpm) at 37°C. An OD_{600} of less than 0.05 was considered to be negative. All drugs used in this experiment were bought from Sigma Chemical Co.

Measurement of energy-dependent drug uptake and drug efflux. To measure the energy-dependent uptake of acriflavine, bacteria were grown overnight in 2 ml of LB with appropriate antibiotics at 37°C. A 25-μl portion of the

overnight culture was added to 10 ml of prewarmed (37°C) LB and grown to an OD_{600} of 0.15. A second dilution to an OD_{600} of 0.005 was then made into prewarmed LB containing 2.5 μg of acriflavine per ml, a concentration determined to be subinhibitory. When the OD_{600} reached 0.25 to 0.3, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma) was added to 100 μM (final concentration). Samples of 5 ml were removed 1, 2, 4, 8, 16, or 32 min after addition of CCCP. Control samples were also taken 1, 3, or 5 min before addition of CCCP. Samples were immediately filtered under vacuum onto GF/F glass microfiber filters (Whatman) and washed with 5 ml of ice-cold 0.1 M LiCl. Filters were dried and then extracted with 1.6 ml of dimethyl sulfoxide at 37°C on a shaker for 12 h. A 1.2-ml portion of dimethyl sulfoxide was removed, centrifuged briefly to remove debris, and analyzed by fluorescence. The relative amount of acriflavine retained in cells was measured by exciting samples at 485 nm, monitoring emission at 501 nm with a luminescence spectrometer (LS-5B; Perkin Elmer), and normalizing data to the total protein concentration. To monitor the energy-dependent efflux of acriflavine from cells, cultures were diluted and grown as described above except that acriflavine was omitted from the medium. At an OD_{600} of 0.1, the cells were removed from the warm room, and all subsequent steps were performed at room temperature. A 50-ml portion of the culture was centrifuged, and the pellet was resuspended in an equal volume of M9 salts containing 100 μM CCCP and 1.25 μg of acriflavine per ml. Cells were incubated for 1 h in the dark on a shaker to deplete ATP reserves and load cells with acriflavine. Cells were then spun down, washed, and resuspended in 50 ml of M9 salts containing 1.25 μg of acriflavine per ml but no CCCP. A mixture of glucose and vitamin B_1 was then added at time zero to achieve final concentrations of 0.2% and 0.5 μg/ml, respectively. The samples were filtered, extracted, and measured by fluorescence as before.

Nucleotide sequence accession number. The GenBank accession number for *acrA* and *acrE* is U00734.

RESULTS

Cloning of the *acrA* locus by complementation. The *acrA* locus is situated between 10 and 11 min on the genetic map of *E. coli* (31). Two isolates from the Kohara bacteriophage λ library, K150 (19B7) and K151 (8C4), span this region (26, 39). Five DNA fragments ranging from 5.5 to 10 kb were subcloned from K150 and K151 into high-copy-number vectors to test for complementation of the *acrA1* allele in strain N43. As judged from previous reports (3) and our data (see below), N43 is susceptible to mitomycin C at 0.1 μg/ml and novobiocin at 5 μg/ml, whereas its isogenic parent W4573 (*acrA*$^+$) is resistant to mitomycin C at 1 μg/ml and novobiocin at 30 μg/ml. Therefore, N43 transformed by these plasmid constructs was tested for its ability to grow on LB agar plates containing mitomycin C at 0.3 μg/ml or novobiocin at 30 μg/ml. As shown in Fig. 1, two of the five DNAs, a 6.5-kb *Bgl*II-*Bgl*II fragment (pUCK151A) and a 7.4-kb *Eco*RV-*Hin*dIII fragment (pBRK151B), both from K151, supported growth of N43 in the presence of otherwise inhibitory concentrations of mitomycin C and novobiocin. A plasmid containing DNA common to these two constructs, the 1.8-kb *Bgl*II-*Eco*RV fragment of pHA, also rescued the drug susceptibility of N43. These results suggest either that the drug resistance determinant of *acrA* resides within this 1.8-kb region or that overexpression of this region on a high-copy-number plasmid can suppress the phenotype of an *acrA* mutant. To confirm that the drug resistance supplied by plasmid pHA was caused by complementation of *acrA* and not by suppression, we cloned a 1.6-kb *Bgl*II-*Nae*I fragment, which contained all but 200 bp of the insert from pHA, into the low-copy-number vector pACYC177 (Fig. 1). The resulting construct, designated pLA, was still able to complement the drug susceptibility of N43, suggesting that *acrA* was indeed encoded by this DNA fragment.

Sequence analysis of the *acrA* locus. The 1.8-kb *Bgl*II-*Eco*RV fragment from pHA was sequenced (nucleotides 3 to 1847; Fig. 2). It contained one intact upstream ORF and one incomplete downstream ORF arranged in parallel orientation. Since this 1.8-kb fragment alone was sufficient to complement the drug-susceptible phenotype of the *acrA* mutant N43, we named the upstream gene *acrA*. This designation is consistent with the subsequent analysis of the site of mutation in strain N43. The *acrA* gene product is predicted to contain 397 amino acids with a molecular weight of 42,000. The first 24 amino acids of AcrA show features typical of a prokaryotic lipoprotein signal peptide, including two positively charged residues (Lys and Arg) near the N terminus, a central hydrophobic domain (Phe-Thr-Pro-Leu-Ala-Val-Val-Leu-Met-Leu), and a C-terminal region which contains the consensus processing site for prelipoprotein precursors (Leu-Thr-Gly-Cys) (10). Since the downstream ORF is probably also involved in the full drug resistance observed in the parental strain W4573 (see below), we extended our sequencing to encompass this ORF. This second ORF encodes a protein of 1,049 amino acids with a molecular weight of 114,000. We named this gene *acrE* (*acrB*, *acrC*, and *acrD* have been previously assigned [1]). A possible rho-independent termination signal was identified about 40 nucleotides downstream of the stop codon of *acrE*. No transcription termination signal was observed in the intergenic region between *acrA* and *acrE*.

Hydropathy plots for AcrA and AcrE are shown in Fig. 3. Except for the potential N-terminal signal peptide, no transmembrane segment is evident in AcrA. On the other hand, AcrE contains several (about 12) highly hydrophobic regions but no potential signal peptide. It is especially interesting that AcrE contains two large hydrophilic domains (amino acids 50 to 350 and 575 to 875) and that the N- and C-terminal halves of AcrE have symmetrical hydropathy profiles (Fig. 3B).

Identification of the *acrA* mutation in N43. We have identified the exact mutation in N43 by PCR amplification and DNA sequencing (Fig. 4). Unexpectedly, when genomic DNA from N43 was used as the template, the amplified product was about 1.3 kb longer than predicted from our sequence, indicating the existence of insertion at or near the 5′ end of *acrA* (Fig. 4A and B). Using various combinations of forward and reverse primers for PCR, we were able to narrow the insertion site down to the region between the primers F3 and R5. Sequencing revealed that an IS*2* element was inserted within the second codon of *acrA* (Fig. 4C). One consequence of such an insertion would be the absence of the *acrA* gene product in N43, which by itself might account for the observed drug susceptibility. It is also possible that this IS*2* insertion has additional polar effects on downstream genes such as *acrE*.

The cellular localization of AcrA and AcrE. The Tn*phoA* method of generating fusions to the *E. coli phoA* gene was originally developed to identify proteins containing membrane translocation or insertion signals (22). Recently, the same approach has also been applied to study the topology of membrane proteins (23). We have analyzed the cellular

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CCA GAT CTC ACT GAA CAA ATC CGA CTT GTC TTT AAA ATG CCA GTA GAT TGC ACC GCG CGT    60
AAC GCC AGC TGC TTT TGC AAT CTC GCC CAG CGA GGT GGA TGA TAC CCC CTG CTG TGA GAA   120
AAG ACG TAG AGC CAC ATC GAG GAT GTG TTG GCG CGT TTC TTG CGC TTC TTG TTT GGT TTT   180
TCG TGC CAT ATG TTC GTG AAT TTA CAG GCG TTA GAT TTA CAT ACA TTT GTG AAT GTA TGT   240
ACC ATA GCA CGA CGA TAA TAT AAA CGC AGC AAT GGG TTT ATT AAC TTT TGA CCA TTG ACC   300
AAT TTG AAA TCG GAC ACT CGA GGT TTA CAT ATG AAC AAA AAC AGA GGG TTT ACG CCT CTG   360
                        *****          Met Asn Lys Asn Arg Gly Phe Thr Pro Leu

GCG GTC GTT CTG ATG CTC TCA GGC AGC TTA GCC CTA ACA GGA TGT GAC GAC AAA CAG GCC   420
Ala Val Val Leu Met Leu Ser Gly Ser Leu Ala Leu Thr Gly Cys Asp Asp Lys Gln Ala

CAA CAA GGT GGC CAG CAG ATG CCC GCC GTT GGC GTA GTA ACA GTC AAA ACT GAA CCT CTG   480
Gln Gln Gly Gly Gln Gln Met Pro Ala Val Gly Val Val Thr Val Lys Thr Glu Pro Leu

CAG ATC ACA ACC GAG CTT CCG GGT CGC ACC AGT GCC TAC CGG ATC GCA GAA GTT CGT CCT   540
Gln Ile Thr Thr Glu Leu Pro Gly Arg Thr Ser Ala Tyr Arg Ile Ala Glu Val Arg Pro

CAA GTT AGC GGG ATT ATC CTG AAG CGT AAT TTC AAA GAA GGT AGC GAC ATC GAA GCA GGT   600
Gln Val Ser Gly Ile Ile Leu Lys Arg Asn Phe Lys Glu Gly Ser Asp Ile Glu Ala Gly

GTC TCT CTC TAT CAG ATT GAT CCT GCG ACC TAT CAG GCG ACA TAC GAC AGT GCG AAA GGT   660
Val Ser Leu Tyr Gln Ile Asp Pro Ala Thr Tyr Gln Ala Thr Tyr Asp Ser Ala Lys Gly

GAT CTG GCG AAA GCC CAG GCT GCA GCC AAT ATC GCG CAA TTG ACG GTG AAT CGT TAT CAG   720
Asp Leu Ala Lys Ala Gln Ala Ala Ala Asn Ile Ala Gln Leu Thr Val Asn Arg Tyr Gln

AAA CTG CTC GGT ACT CAG TAC ATC AGT AAG CAA GAG TAC GAT CAG GCT CTG GCT GAT GCG   780
Lys Leu Leu Gly Thr Gln Tyr Ile Ser Lys Gln Glu Tyr Asp Gln Ala Leu Ala Asp Ala

CAA CAG GCG AAT GCT GCG GTA ACT GCG GCG AAA GCT GCC GTT GAA ACT GCG CGG ATC AAT   840
Gln Gln Ala Asn Ala Ala Val Thr Ala Ala Lys Ala Ala Val Glu Thr Ala Arg Ile Asn

CTG GCT TAC ACC AAA GTC ACC TCT CCG ATT AGC GGT CGC ATT GGT AAG TCG AAC GTG ACG   900
Leu Ala Tyr Thr Lys Val Thr Ser Pro Ile Ser Gly Arg Ile Gly Lys Ser Asn Val Thr

GAA GGC GCA TTG GTA CAG AAC GGT CAG GCG ACT GCG CTG GCA ACC GTG CAG CAA CTT GAT   960
Glu Gly Ala Leu Val Gln Asn Gly Gln Ala Thr Ala Leu Ala Thr Val Gln Gln Leu Asp

CCG ATC TAC GTT GAT GTG ACC CAG TCC AGC AAC GAC TTC CTG CGC CTG AAA CAG GAA CTG  1020
Pro Ile Tyr Val Asp Val Thr Gln Ser Ser Asn Asp Phe Leu Arg Leu Lys Gln Glu Leu

GCG AAT GGC ACG CTG AAA CAA GAG AAC GGC AAA GCC AAA GTG TCA CTG ATC ACC AGT GAC  1080
Ala Asn Gly Thr Leu Lys Gln Glu Asn Gly Lys Ala Lys Val Ser Leu Ile Thr Ser Asp

GGC ATT AAG TTC CCG CAG GAC GGT ACG CTG GAA TTC TCT GAC GTT ACC GTT GAT CAG ACC  1140
Gly Ile Lys Phe Pro Gln Asp Gly Thr Leu Glu Phe Ser Asp Val Thr Val Asp Gln Thr

ACT GGG TCT ATC ACC CTA CGC GCT ATC TTC CCG AAC CCG GAT CAC ACT CTG CTG CCG GGT  1200
Thr Gly Ser Ile Thr Leu Arg Ala Ile Phe Pro Asn Pro Asp His Thr Leu Leu Pro Gly

ATG TTC GTG CGC GCA CGT CTG GAA GAA GGG CTT AAT CCA AAC GCT ATT TTA GTC CCG CAA  1260
Met Phe Val Arg Ala Arg Leu Glu Glu Gly Leu Asn Pro Asn Ala Ile Leu Val Pro Gln

CAG GGC GTA ACC CGT ACG CCG CGT GGC GAT GCC ACC GTA CTG GTA GTT GGC GCG GAT GAC  1320
Gln Gly Val Thr Arg Thr Pro Arg Gly Asp Ala Thr Val Leu Val Val Gly Ala Asp Asp

AAA GTG GAA ACC CGT CCG ATC GTT GCA AGC CAG GCT ATT GGC GAT AAG TGG CTG GTG ACA  1380
Lys Val Glu Thr Arg Pro Ile Val Ala Ser Gln Ala Ile Gly Asp Lys Trp Leu Val Thr

GAA GGT CTG AAA GCA GGC GAT CGC GTA GTA ATA AGT GGG CTG CAG AAA GTG CGT CCT GGT  1440
Glu Gly Leu Lys Ala Gly Asp Arg Val Val Ile Ser Gly Leu Gln Lys Val Arg Pro Gly
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FIG. 2. Nucleotide sequences of *acrA*, *acrE*, and their flanking regions. *acrA* is located between bases 331 and 1521; *acrE* is located between bases 1547 and 4693. Start codons and stop codons are printed in bold type. The suggested ribosomal binding sites are marked with asterisks. Arrows represent the rho-independent termination signal. The underlined hexanucleotides are some recognition sites for restriction enzymes used in this study: AGATCT (*Bgl*II), GCCGGC (*Nae*I), and GATATC (*Eco*RV).

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GTC CAG GTA AAA GCA CAA GAA GTT ACC GCT GAT AAT AAC CAG CAA GCC GCA AGC GGT GCT  1500
Val Gln Val Lys Ala Gln Glu Val Thr Ala Asp Asn Asn Gln Gln Ala Ala Ser Gly Ala

CAG CCT GAA CAG TCC AAG TCT TAA  C TTA AAC AGG AGC CGT TAA GAC  ATG CCT AAT TTC  1558
Gln Pro Glu Gln Ser Lys Ser ---            ******              Met Pro Asn Phe

TTT ATC GAT CGC CCG ATT TTT GCG TGG GTG ATC GCC ATT ATC ATC ATG TTG GCA GGG GGG  1618
Phe Ile Asp Arg Pro Ile Phe Ala Trp Val Ile Ala Ile Ile Ile Met Leu Ala Gly Gly

CTG GCG ATC CTC AAA CTG CCG GTG GCG CAA TAT CCT ACG ATT GCA CCG CCG GCA GTA ACG  1678
Leu Ala Ile Leu Lys Leu Pro Val Ala Gln Tyr Pro Thr Ile Ala Pro Pro Ala Val Thr

ATC TCC GCC TCC TAC CCC GGC GCT GAT GCG AAA ACA GTG CAG GAC ACG GTG ACA CAG GTT  1738
Ile Ser Ala Ser Tyr Pro Gly Ala Asp Ala Lys Thr Val Gln Asp Thr Val Thr Gln Val

ATC GAA CAG AAT ATG AAC GGT ATC GAT AAC CTG ATG TAC ATG TCC TCT AAC AGT GAC TCC  1798
Ile Glu Gln Asn Met Asn Gly Ile Asp Asn Leu Met Tyr Met Ser Ser Asn Ser Asp Ser

ACG GGT ACC GTG CAG ATC ACC CTG ACC TTT GAG TCT GGT ACT GAT GCG GAT ATC GCG CAG  1858
Thr Gly Thr Val Gln Ile Thr Leu Thr Phe Glu Ser Gly Thr Asp Ala Asp Ile Ala Gln

GTT CAG GTG CAG AAC AAA CTG CAG CTG GCG ATG CCG TTG CTG CCG CAA GAA GTT CAG CAG  1918
Val Gln Val Gln Asn Lys Leu Gln Leu Ala Met Pro Leu Leu Pro Gln Glu Val Gln Gln

CAA GGG GTG AGC GTT GAG AAA TCA TCC AGC AGC TTC CTG ATG GTT GTC GGC GTT ATC AAC  1978
Gln Gly Val Ser Val Glu Lys Ser Ser Ser Ser Phe Leu Met Val Val Gly Val Ile Asn

ACC GAT GGC ACC ATG ACG CAG GAG GAT ATC TCC GAC TAC GTG GCG GCG AAT ATG AAA GAT  2038
Thr Asp Gly Thr Met Thr Gln Glu Asp Ile Ser Asp Tyr Val Ala Ala Asn Met Lys Asp

GCC ATC AGC CGT ACG TCG GGC GTG GGT GAT GTT CAG TTG TTC GGT TCA CAG TAC GCG ATG  2098
Ala Ile Ser Arg Thr Ser Gly Val Gly Asp Val Gln Leu Phe Gly Ser Gln Tyr Ala Met

CGT ATC TGG ATG AAC CCG AAT GAG CTG AAC AAA TTC CAG CTA ACG CCG GTT GAT GTC ATT  2158
Arg Ile Trp Met Asn Pro Asn Glu Leu Asn Lys Phe Gln Leu Thr Pro Val Asp Val Ile

ACC GCC ATC AAA GCG CAG AAC GCC CAG GTT GCG GCG GGT CAG CTC GGT GGT ACG CCG CCG  2218
Thr Ala Ile Lys Ala Gln Asn Ala Gln Val Ala Ala Gly Gln Leu Gly Gly Thr Pro Pro

GTG AAA GGC CAA CAG CTT AAC GCC TCT ATT ATT GCT CAG ACG CGT CTG ACC TCT ACT GAA  2278
Val Lys Gly Gln Gln Leu Asn Ala Ser Ile Ile Ala Gln Thr Arg Leu Thr Ser Thr Glu

GAG TTC GGC AAA ATC CTG CTG AAA GTG AAT CAG GAT GGT TCC CGC GTG CTG CTG CGT GAC  2338
Glu Phe Gly Lys Ile Leu Leu Lys Val Asn Gln Asp Gly Ser Arg Val Leu Leu Arg Asp

GTC GCG AAG ATT GAG CTG GGT GGT GAG AAC TAC GAC ATC ATC GCA GAG TTT AAC GGC CAA  2398
Val Ala Lys Ile Glu Leu Gly Gly Glu Asn Tyr Asp Ile Ile Ala Glu Phe Asn Gly Gln

CCG GCT TCC GGT CTG GGG ATC AAG CTG GCG ACC GGT GCA AAC GCG CTG GAT ACC GCT GCG  2458
Pro Ala Ser Gly Leu Gly Ile Lys Leu Ala Thr Gly Ala Asn Ala Leu Asp Thr Ala Ala

GCA ATC CGT GCT GAA CTG GCG AAG ATG GAA CCG TTC TTC CCG TCG GGT CTG AAA ATT GTT  2518
Ala Ile Arg Ala Glu Leu Ala Lys Met Glu Pro Phe Phe Pro Ser Gly Leu Lys Ile Val

TAC CCA TAC GAC ACC ACG CCG TTC GTG AAA ATC TCT ATT CAC GAA GTG GTT AAA ACG CTG  2578
Tyr Pro Tyr Asp Thr Thr Pro Phe Val Lys Ile Ser Ile His Glu Val Val Lys Thr Leu

GTC GAA GCG ATC ATC CTC GTG TTC CTG GTT ATG TAT CTG TTC CTG CAG AAC TTC CGC GCG  2638
Val Glu Ala Ile Ile Leu Val Phe Leu Val Met Tyr Leu Phe Leu Gln Asn Phe Arg Ala

ACG TTG ATT CCG ACC ATT GCC GTA CCG GTG GTA TTG CTC GGG ACC TTT GCC GTC CTT GCC  2698
Thr Leu Ile Pro Thr Ile Ala Val Pro Val Val Leu Leu Gly Thr Phe Ala Val Leu Ala

GCC TTT GGC TTC TCG ATA AAC ACG CTA ACA ATG TTC GGG ATG GTG CTC GCC ATC GGC CTG  2758
Ala Phe Gly Phe Ser Ile Asn Thr Leu Thr Met Phe Gly Met Val Leu Ala Ile Gly Leu
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FIG. 2—*Continued.*

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TTG GTG GAT GAC GCC ATC GTT GTG GTA GAA AAC GTT GAG CGT GTT ATG GCG GAA GAA GGT  2818
Leu Val Asp Asp Ala Ile Val Val Val Glu Asn Val Glu Arg Val Met Ala Glu Glu Gly

TTG CCG CCA AAA GAA GCT ACC CGT AAG TCG ATG GGG CAG ATT CAG GGC GCT CTG GTC GGT  2878
Leu Pro Pro Lys Glu Ala Thr Arg Lys Ser Met Gly Gln Ile Gln Gly Ala Leu Val Gly

ATC GCG ATG GTA CTG TCG GCG GTA TTC GTA CCG ATG GCC TTC TTT GGC GGT TCT ACT GGT  2938
Ile Ala Met Val Leu Ser Ala Val Phe Val Pro Met Ala Phe Phe Gly Gly Ser Thr Gly

GCT ATC TAT CGT CAG TTC TCT ATT ACC ATT GTT TCA GCA ATG GCG CTG TCG GTA CTG GTG  2998
Ala Ile Tyr Arg Gln Phe Ser Ile Thr Ile Val Ser Ala Met Ala Leu Ser Val Leu Val

GCG TTG ATC CTG ACT CCA GCT CTT TGT GCC ACC ATG CTG AAA CCG ATT GCC AAA GGC GAT  3058
Ala Leu Ile Leu Thr Pro Ala Leu Cys Ala Thr Met Leu Lys Pro Ile Ala Lys Gly Asp

CAC GGG GAA GGT AAA AAA GGC TTC TTC GGC TGG TTT AAC CGC ATG TTC GAG AAG AGC ACG  3118
His Gly Glu Gly Lys Lys Gly Phe Phe Gly Trp Phe Asn Arg Met Phe Glu Lys Ser Thr

CAC CAC TAC ACC GAC AGC GTA GGC GGT ATT CTG CGC AGT ACG GGG CGT TAC CTG GTG CTG  3178
His His Tyr Thr Asp Ser Val Gly Gly Ile Leu Arg Ser Thr Gly Arg Tyr Leu Val Leu

TAT CTG ATC ATC GTG GTC GGC ATG GCC TAT CTG TTC GTG CGT CTG CCA AGC TCC TTC TTG  3238
Tyr Leu Ile Ile Val Val Gly Met Ala Tyr Leu Phe Val Arg Leu Pro Ser Ser Phe Leu

CCA GAT GAG GAC CAG GGC GTG TTT ATG ACC ATG GTT CAG CTG CCA GCA GGT GCA ACG CAG  3298
Pro Asp Glu Asp Gln Gly Val Phe Met Thr Met Val Gln Leu Pro Ala Gly Ala Thr Gln

GAA CGT ACA CAG AAA GTG CTC AAT GAG GTA ACG CAT TAC TAT CTG ACC AAA GAA AAG AAC  3358
Glu Arg Thr Gln Lys Val Leu Asn Glu Val Thr His Tyr Tyr Leu Thr Lys Glu Lys Asn

AAC GTT GAG TCG GTG TTC GCC GTT AAC GGC TTC GGC TTT GCG GGA CGT GGT CAG AAT ACC  3418
Asn Val Glu Ser Val Phe Ala Val Asn Gly Phe Gly Phe Ala Gly Arg Gly Gln Asn Thr

GGT ATT GCG TTC GTT TCC TTG AAG GAC TGG GCC GAT CGT CCG GGC GAA GAA AAC AAA GTT  3478
Gly Ile Ala Phe Val Ser Leu Lys Asp Trp Ala Asp Arg Pro Gly Glu Glu Asn Lys Val

GAA GCG ATT ACC ATG CGT GCA ACA CGC GCT TTC TCG CAA ATC AAA GAT GCG ATG GTT TTC  3538
Glu Ala Ile Thr Met Arg Ala Thr Arg Ala Phe Ser Gln Ile Lys Asp Ala Met Val Phe

GCC TTT AAC CTG CCC GCA ATC GTG GAA CTG GGT ACT GCA ACC GGC TTT GAC TTT GAG CTG  3598
Ala Phe Asn Leu Pro Ala Ile Val Glu Leu Gly Thr Ala Thr Gly Phe Asp Phe Glu Leu

ATT GAC CAG GCT GGC CTT GGT CAC GAA AAA CTG ACT CAG GCG CGT AAC CAG TTG CTT GCA  3658
Ile Asp Gln Ala Gly Leu Gly His Glu Lys Leu Thr Gln Ala Arg Asn Gln Leu Leu Ala

GAA GCA GCG AAG CAC CCT GAT ATG TTG ACC AGC GTA CGT CCA AAC GGT CTG GAA GAT ACC  3718
Glu Ala Ala Lys His Pro Asp Met Leu Thr Ser Val Arg Pro Asn Gly Leu Glu Asp Thr

CCG CAG TTT AAG ATT GAT ATC GAC CAG GAA AAA GCG CAG GCG CTG GGT GTT TCT ATC AAC  3778
Pro Gln Phe Lys Ile Asp Ile Asp Gln Glu Lys Ala Gln Ala Leu Gly Val Ser Ile Asn

GAC ATT AAC ACC ACT CTG GGC GCT GCA TGG GGC GGC AGC TAT GTG AAC GAC TTT ATC GAC  3838
Asp Ile Asn Thr Thr Leu Gly Ala Ala Trp Gly Gly Ser Tyr Val Asn Asp Phe Ile Asp

CGC GGT CGT GTG AAG AAA GTT TAT GTC ATG TCA GAA GCG AAA TAC CGT ATG CTG CCG GAT  3898
Arg Gly Arg Val Lys Lys Val Tyr Val Met Ser Glu Ala Lys Tyr Arg Met Leu Pro Asp

GAT ATC GGC GAC TGG TAT GTT CGT GCT GCT GAT GGT CAG ATG GTG CCA TTC TCG GCG TTC  3958
Asp Ile Gly Asp Trp Tyr Val Arg Ala Ala Asp Gly Gln Met Val Pro Phe Ser Ala Phe

TCC TCT TCT CGT TGG GAG TAC GGT TCG CCG CGT CTG GAA CGT TAC AAC GGC CTG CCA TCC  4018
Ser Ser Ser Arg Trp Glu Tyr Gly Ser Pro Arg Leu Glu Arg Tyr Asn Gly Leu Pro Ser
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FIG. 2—*Continued.*

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ATG GAA ATC TTA GGC CAG GCG GCA CCG GGT AAA AGT ACC GGT GAA GCA ATG GAG CTG ATG  4078
Met Glu Ile Leu Gly Gln Ala Ala Pro Gly Lys Ser Thr Gly Glu Ala Met Glu Leu Met

GAA CAA CTG GCG AGC AAA CTG CCT ACC GGT GTT GGC TAT GAC TGG ACG GGG ATG TCC TAT  4138
Glu Gln Leu Ala Ser Lys Leu Pro Thr Gly Val Gly Tyr Asp Trp Thr Gly Met Ser Tyr

CAG GAA CGT CTC TCC GGC AAC CAG GCA CCT TCA CTG TAC GCG ATT TCG TTG ATT GTC GTG  4198
Gln Glu Arg Leu Ser Gly Asn Gln Ala Pro Ser Leu Tyr Ala Ile Ser Leu Ile Val Val

TTC CTG TGT CTG GCG GCG CTG TAC GAG AGC TGG TCG ATT CCG TTC TCC GTT ATG CTG GTC  4258
Phe Leu Cys Leu Ala Ala Leu Tyr Glu Ser Trp Ser Ile Pro Phe Ser Val Met Leu Val

GTT CCG CTG GGG GTT ATC GGT GCG TTG CTG GCT GCC ACC TTC CGT GGC CTG ACC AAT GAC  4318
Val Pro Leu Gly Val Ile Gly Ala Leu Leu Ala Ala Thr Phe Arg Gly Leu Thr Asn Asp

GTT TAC TTC CAG GTA GGC CTG CTC ACA ACC ATT GGG TTG TCG GCG AAG AAC GCG ATC CTT  4378
Val Tyr Phe Gln Val Gly Leu Leu Thr Thr Ile Gly Leu Ser Ala Lys Asn Ala Ile Leu

ATC GTC GAA TTC GCC AAA GAC TTG ATG GAT AAA GAA GGT AAA GGT CTG ATT GAA GCG ACG  4438
Ile Val Glu Phe Ala Lys Asp Leu Met Asp Lys Glu Gly Lys Gly Leu Ile Glu Ala Thr

CTT GAT GCG GTG CGG ATG CGT TTA CGT CCG ATC CTG ATG ACC TCG CTG GCG TTT ATC CTC  4498
Leu Asp Ala Val Arg Met Arg Leu Arg Pro Ile Leu Met Thr Ser Leu Ala Phe Ile Leu

GGC GTT ATG CCG CTG GTT ATC AGT ACT GGT GCT GGT TCC GGC GCG CAG AAC GCA GTA GGT  4558
Gly Val Met Pro Leu Val Ile Ser Thr Gly Ala Gly Ser Gly Ala Gln Asn Ala Val Gly

ACC GGT GTA ATG GGC GGG ATG GTG ACC GCA ACG GTA CTG GCA ATC TTC TTC GTT CCG GTA  4618
Thr Gly Val Met Gly Gly Met Val Thr Ala Thr Val Leu Ala Ile Phe Phe Val Pro Val

TTC TTT GTG GTG GTT CGC CGC CGC TTT AGC CGC AAG AAT GAA GAT ATC GAG CAC AGC CAT  4678
Phe Phe Val Val Val Arg Arg Arg Phe Ser Arg Lys Asn Glu Asp Ile Glu His Ser His

ACT GTC GAT CAT CAT TGA TAC AAC GTG TAA TCA CTA AGG CCG CGT AAG CGG CCT TTT TTA  4738
Thr Val Asp His His ---                          ------>  <------

TGC ATA ACC TAC GAA CA  4759
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FIG. 2—*Continued.*

localizations of AcrA and AcrE by this method. After transferring λ::Tn*phoA* to CC118 carrying pHA, we selected blue colonies on plates which contained the phosphatase indicator dye 5-bromo-3-chloroindolyl phosphate. CC118 is an *E. coli* strain in which the chromosomal *phoA* gene has been deleted (22). Selection of Tn*phoA* fusion was carried out according to previously described procedures (22) so that blue colonies were generated only when the cloned 1.8-kb *Bgl*II-*Eco*RV fragment provided an export signal. Restriction analysis of plasmid DNA from 40 blue colonies revealed 11 different constructs. Subsequent sequencing showed that 10 of them contained Tn*phoA* fused in frame with *acrA* (Table 1) and further indicated that a significant portion of mature AcrA resides in the periplasm. Interestingly, none of the 10 constructs contained a Tn*phoA* insertion between amino acids 197 and 370 in AcrA. We obtained only one in-frame Tn*phoA* fusion with AcrE that showed high phosphatase activity, probably as a result of the fact that only the first 101 amino acids of AcrE were coded on pHA. Nevertheless, this result confirms that AcrE is expressed in *E. coli* and suggests that at least part of AcrE resides in the periplasm. These data, combined with the hydropathy plot and the lack of detectable signal peptide, make us tentatively assume that *acrE* may encode an integral membrane protein with multiple (about 12) transmembrane domains.

Homology between AcrAE and EnvCD in *E. coli*. An amino acid sequence comparison with GenBank files revealed that AcrA and AcrE share significant sequence similarity with two previously studied *E. coli* gene products, EnvC and EnvD (encoded by *envC* and *envD*, mapped at 73 min of the physical map [16]). AcrA shared 65% amino acid identity with EnvC. Though AcrE had considerable stretches of similarity with the published EnvD sequence, the N-terminal 68 amino acids of AcrE lacked a comparable region in EnvD. Several discrepancies were also present in other regions. These inconsistencies led us to resequence the regions in question, starting from Kohara phage K530 as a source of DNA. We discovered several errors in the original sequence reported by Klein et al. (15) (Table 2). Using the revised *envD* sequence, we found AcrE and EnvD to be 77% identical (Fig. 5). These strong homologies imply similar structures and functions for AcrAE and EnvCD. Interestingly, EnvC has been shown to be processed posttranslationally, probably as a result of removal of the N-terminal signal peptide (15). *E. coli* PM61, which contains an unidentified mutation in the *envCD* locus, was also reported to be

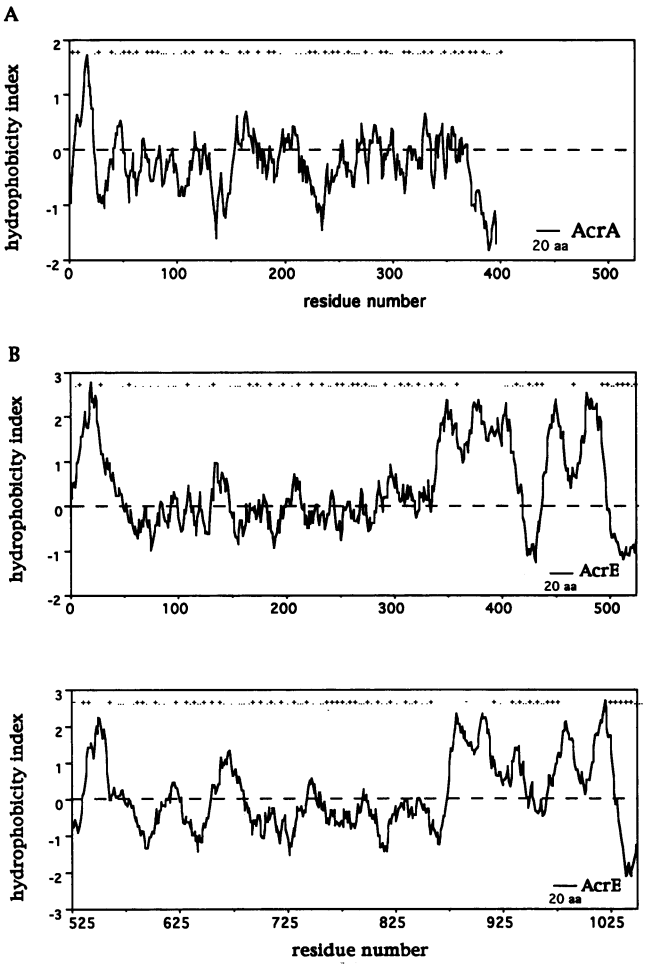

FIG. 3. Hydropathy profiles of AcrA and AcrE. The hydrophobicity scale was chosen according to Kyte and Doolittle (17a), and the window size was 19 residues. The distribution of charged residues is indicated on the top (+ means Arg or Lys; . means Asp or Glu). (A) Except for the most N-terminal signal peptide, no potential trans-inner membrane segments are evident in AcrA. (B) AcrE contains multiple (about 12) highly hydrophobic regions. Notice the striking similarities between the N- and C-terminal halves of AcrE.

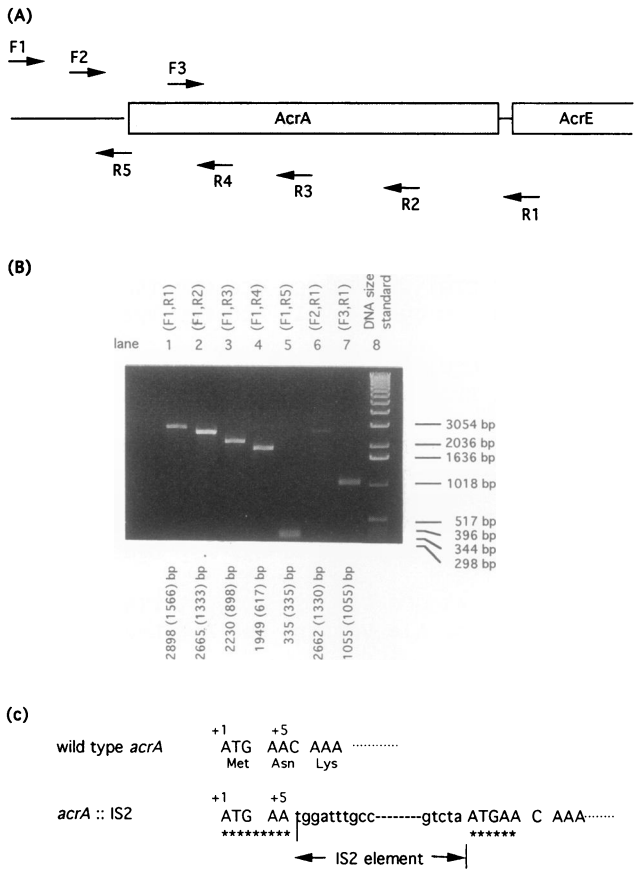

FIG. 4. Characterization of the *acr* mutation in N43 by PCR and sequencing. (A) Primers used in PCR. Their sites, relative to +1 at the A residue of the initial methionine of AcrA, are as follows: F1, −327 to −308; F2, −91 to −72; F3, 185 to 203; R1, 1239 to 1218; R2, 1006 to 986; R3, 571 to 552; R4, 290 to 271; and R5, 8 to −12. (B) The PCR products from different combinations of primers. The 1-kb DNA ladder (from GIBCO BRL) was used as the size standard (lane 8). The expected (in parentheses) versus found lengths of the PCR products (lane 1 to 7) are indicated. Only lanes 5 and 7 showed the products with the expected lengths, indicating the presence of an insertion between R5 and F3. (C) An IS2 element is inserted between the +5 and +6 sites of the *acrA* gene. Lowercase letters represent the 1,327-bp nucleotide sequence of the IS2 element (9). Asterisks denote the duplication of a 5-bp target site (ATGAA).

hypersusceptible to basic dyes, detergents, and antibiotics (16).

Drug susceptibilities of *acrAE* and *envCD* mutants. To understand the roles that AcrAE and EnvCD play in affecting drug susceptibilities, the MICs of various drugs have been determined and compared for both mutants (N43 and PM61) and their corresponding parental strains (W4573 and P678S^R) (Table 3). We point out here that P678, rather than P678S^R, is the isogenic parent of PM61 (12). P678S^R is derived from P678, and they differ in that P678S^R carries an additional mutation at the *mtl* locus. *mtl* is not known to affect any drug susceptibility in *E. coli*.

Both mutants show hypersusceptibility to mitomycin C, erythromycin, and fusidic acid (>15-fold). Interestingly, N43 is much more susceptible to ethidium bromide and novobiocin than is PM61. On the other hand, PM61 is far more sensitive to actinomycin D, vancomycin, and penicillin G than is N43.

A model to explain the drug susceptibility of N43 and PM61 is that *acrAE* and *envCD* encode two transmembrane drug efflux pumps. This model is tempting since no apparent defect of the outer membrane was reported in N43 or PM61. To test this model, we have studied acriflavine uptake by intact cells in the absence and presence of CCCP. CCCP is a potent inhibitor that dissipates the electrochemical proton gradient. Since drug pumps use either the proton motive force or ATP as an energy source to expel drugs, CCCP will abolish their ability to pump drug molecules against a concentration gradient (19). If a concentration gradient across the cell membrane has been generated as the result of an energy-dependent pumping process, addition of CCCP will lead to rapid influx of the drug into the cell. As Fig. 6 shows, prior to the addition of CCCP, a higher level of acriflavine (about fourfold) was accumulated in N43 than in the wild type, W4573, in the presence of the same amount of acriflavine. This observation is consistent with the acriflavine-susceptible phenotype of N43. After addition of 100 μM

TABLE 1. Fusion junctions of AcrA-Tn*phoA* on plasmid pHA and their alkaline phosphatase activities in strain CC118

Plasmid	Junction in AcrA (amino acid no.)[a]	Alkaline phosphatase activity (U/OD_{600})
pHA::Tn*phoA*49	49	2,056
pHA::Tn*phoA*68	68	1,388
pHA::Tn*phoA*91	91	1,203
pHA::Tn*phoA*110	110	983
pHA::Tn*phoA*117	117	1,042
pHA::Tn*phoA*160	160	1,046
pHA::Tn*phoA*176	176	1,045
pHA::Tn*phoA*196	196	509
pHA::Tn*phoA*371	371	1,775
pHA::Tn*phoA*374	374	2,085
pHA::Tn*phoA*30	30[b]	1,540
None		<5[c]

[a] The junction represents the amino acid residue within AcrA where Tn*phoA* is inserted. The fusion protein will then contain the residues before the junction joined to alkaline phosphatase.
[b] In this construct, Tn*phoA* is fused within the N-terminal portion of AcrE, not AcrA.
[c] Background level of alkaline phosphatase activity in strain CC118.

CCCP, rapid accumulation of acriflavine was observed in both N43 and W4573. Therefore, both N43 and W4573 possess the ability to expel acriflavine against a concentration gradient. We have also measured the kinetics of acriflavine efflux from N43 and W4573. Cells were loaded with acriflavine in the presence of CCCP. After CCCP was washed from cells, there was a rapid acriflavine efflux from the cells. However, we could not find large differences in efflux rates between the two strains (data not shown).

AcrE may also be involved in drug susceptibilities. The genes *envC* and *envD* have been shown to be on the same operon, and both gene products are required to complement the drug susceptibility in PM61 (16). This finding suggests the possibility of a similar situation for *acrA* and *acrE*. To test this idea, we repeated the MIC experiments with novobiocin and ethidium bromide by using pLA, pHA, and pUC151A (Table 4). Although the MICs for N43 harboring either pLA or pHA were comparable with those found in W4573, we did observe that they grew more slowly than the wild type at higher concentrations of these drugs (data not shown). Furthermore, under the same growth conditions, more acriflavine was found accumulated inside N43 in the presence of pHA than in the presence of pUC151A (data not shown). Finally, N43 harboring pUC151A was more resistant, albeit slightly, to novobiocin and ethidium bromide than was W4573. These results suggest a possible role of *acrE* in drug resistance. Plasmids containing only *acrA* may partially complement the drug susceptibility of N43 if low levels of AcrE are synthesized in the mutant, either from an intrinsic or hybrid promoter within IS2 (13) or from an incomplete block of upstream transcription by IS2. Although it seems likely that AcrE is involved in affecting drug susceptibilities, we would like to emphasize here that support from more definitive experiments such as the construction of *acrE* null mutant is necessary to confirm this point. It is also not known whether a gene or genes other than *acrA* and *acrE* are involved in drug susceptibilities.

TABLE 2. Partial revision of the *envD* sequence[a]

Nucleotide no.[b]	Change or changes
1367	Eliminate C
1371	Add G after
1406	Add C after
2253	Add T after
2262	Add G after
2449	Add G after
2507	A to G
2508	G to A
2509	G to T
2510	T to A
2512	C to G
2513	G to C
2665	C to T
2698	C to G
3602	Eliminate C
3616	Add C after
3777	Add T after
3789	Add C after
3799	Add T after

[a] The nucleotide sequence of *envD* was originally reported by Klein et al. (15). We resequenced only the regions from nucleotides 1265 to 1460, 1639 to 1936, 2236 to 2541, 2605 to 2842, 2909 to 3247, 3416 to 3683, and 3756 to 3886.
[b] According to the *envD* sequence deposited in GenBank under accession number X57948.

DISCUSSION

A complementation assay has been used to clone the *acrA* locus, responsible for the drug hypersusceptibility observed in *E. coli* N43. DNA sequencing revealed two ORFs whose genes have been named *acrA* and *acrE*. The *acrA* locus is the site of an insertion by IS2 in N43, the prototypic *acrA* mutant strain. Sequence analysis and Tn*phoA* fusion studies suggest that *acrA* encodes a lipoprotein with most of the mature portion in the periplasm and that *acrE* may encode a protein with multiple transmembrane segments. The second residue in the mature AcrA polypeptide is predicted to be aspartate (Asp), if processing by signal peptidase II occurs as we have suggested. Yamaguchi et al. (44) have proposed that the second residue on a mature lipoprotein functions as a sorting signal for localization to the outer or inner bacterial membrane. According to this hypothesis, an Asp residue at the second position should direct AcrA to the inner membrane. Determination of whether AcrA is an inner membrane lipoprotein awaits more rigorous studies that include metabolic labeling in the presence and absence of globomycin and subcellular fractionation. Similarly, more detailed studies by using additional Tn*phoA* fusions are required to determine the topology of AcrE unambiguously.

Although we isolated 10 in-frame *acrA*::Tn*phoA* fusions, we did not obtain any between residues 197 and 370 of AcrA. Active fusion sites were present at both the N- and C-terminal sides of this region, however. This region constitutes about 50% of the length of AcrA, and the hydropathy plot of AcrA does not reveal any obvious transmembrane segments (Fig. 3A and Table 1). One possible explanation is that the sequence bias for Tn*phoA* transposition discriminates against insertion between residues 197 and 370. Another possibility is that the enzymatic activity of PhoA fused to this particular region of AcrA is low because of its cellular localization. Future work, such as making the Tn*phoA* fusions within this region by PCR, would clarify this issue.

The sequence similarities between AcrAE and EnvCD are striking and suggest a recent gene duplication event. Most likely, these genes evolved from the same ancestor and subsequently diverged in sequence and function. The hydropathy profile of AcrE predicts a 12-transmembrane polypeptide with a symmetrical hydrophobicity pattern between its N- and C-terminal halves (Fig. 3B). Both of these

A

```
AcrA:     1 mnknrgftpLavvlmLsgslaltGCdDKqaqqggqqmPaVgVvtVKTePLqitTELPGRTs
                        |     |        || ||          | | |   ||| ||   |||||||
EnvC:     1  mtkharffLlpsfiLisaaliaGCnDKgeekahvgePqVtVhiVKTaPLevkTELPGRTn

consensus       m--------L-----L-------GC-DK---------P-V-V--VKT-PL---TELPGRT-


AcrA:    62 AYRIAEVRPQVSGIiLkRNFkEGSDieAGvSLYQIDPATYQAtYDSAKGdLAKaqAAAnIA
            |||||||||||||| | ||| ||||  || ||||||||||||| |||||| |||  ||| ||
EnvC:    61 AYRIAEVRPQVSGIvLnRNFtEGSDvqAGqSLYQIDPATYQAnYDSAKGeLAKseAAAaIA

consensus       AYRIAEVRPQVSGI-L-RNF-EGSD--AG-SLYQIDPATYQA-YDSAKG-LAK--AAA-IA


AcrA:   123 qLTVnRYqkLlGTqYISkQEYDQAlADAqQAnAAVtAAKAaVEtARINLAYTKVTsPISGR
             ||| ||  | || ||| |||||| ||| || ||| |||| || ||||||||||| |||||
EnvC:   122 hLTVkRYvpLvGTkYISqQEYDQAiADArQAdAAViAAKAtVEsARINLAYTKVTaPISGR

consensus       -LTV-RY--L-GT-YIS-QEYDQA-ADA-QA-AAV-AAKA-VE-ARINLAYTKVT-PISGR


AcrA:   184 IGKSnVTEgaLvqnGqatalatvqqldpiyVDVTQSSNDFlRLKQelanGtLkqENgkakV
            |||| |||  |   |               |||||||||| ||||    | |  ||    |
EnvC:   183 IGKStVTEalLslmGkrlnwrlssssilstVDVTQSSNDFmRLKQsveqGnLhkENatsnV

consensus       IGKS-VTE--L---G--------------VDVTQSSNDF-RLKQ----G-L--EN----V


AcrA:   245 sLitsdGikfPqdGTLeFSDVTVDqtTGSITLRAiFPNPdHTLLPGMFVRARleEGlnPnA
             |    |   |  ||| |||||||  |||||||| |||| ||||||||||||  ||  | |
EnvC:   244 eLvmenGqtyPlkGTLqFSDVTVDesTGSITLRAvFPNPqHTLLPGMFVRARidEGvqPdA

consensus       -L----G---P--GTL-FSDVTVD--TGSITLRA-FPNP-HTLLPGMFVRAR--EG--P-A


AcrA:   306 ILvPQQGVTrTPRGDATVLvVgaddkVEtRPiVASQAIGDKWLvtEGLKaGDrVviSGLQK
            || |||||| |||||||| |     || || ||||||||||  |||| || |  |||||
EnvC:   305 ILiPQQGVT TPRGDATVLiVndksqVEaRPvVASQAIGDKWLisEGLKsGDqVivSGLQK

consensus       IL-PQQGVT TPRGDATVL-V-----VE-RP-VASQAIGDKWL--EGLK-GD-V--SGLQK


AcrA:   367 vRPGvQVKAqevtadnnqqaasgaqpeqSKs.
             ||| ||||                   ||
EnvC:   365 aRPGeQVKA          ttdtpadtaSK.

consensus       -RPG-QVKA          ---------SK
```

FIG. 5. Amino acid sequence similarities between AcrA and EnvC (A) and between AcrE and EnvD (B). The identical amino acids in each sequence (capital letters) are connected by vertical lines and noted as the consensus. AcrA-EnvC and AcrE-EnvD share 65 and 77% identity, respectively. The underlined parts of EnvD represent the amino acid residues deduced from our resequenced regions of *envD* (also see Table 2).

characteristics are common to proteins which function as transmembrane transporters (11). It remains to be shown whether AcrE (or EnvD) is a transporter of some kind, what the role of AcrA (or EnvC) is in the transport process, and whether there are other, unidentified components involved. If AcrE and EnvD are transporters, one feature which makes them unique from all other prokaryotic transporters is the existence of two large hydrophilic domains (amino acids 50 to 350 and 575 to 875).

The level of acriflavine found in the wild-type strain in comparison with the fourfold-higher level in an *acrA* mutant (Fig. 6) can be explained in at least four ways. (i) This difference could be due to the lower inner membrane permeability in the wild type. We think that this is unlikely, since the diffusion rates of most hydrophobic reagents across the inner membrane are rather rapid (36, 42). (ii) This difference could be the result of lower outer membrane permeability in the wild-type strain. Although this possibility cannot yet be rigorously excluded, the data do not appear to favor this model. First, the entry of acriflavine into the CCCP-treated cells was quite rapid (Fig. 6). This finding is consistent with the recent data (36) that gram-negative

B

```
AcrE:     1 MpNFFIdRPIFAWViAIIiMlAGgLAILkLPVAQYPTIAPPAVtiSAsYPGADAkTVQDTV
EnvD:     1 MaNFFIrRPIFAWVlAIIlMmAGaLAILqLPVAQYPTIAPPAVsvSAnYPGADAqTVQDTV
consensus   M-NFFI-RPIFAWV-AII-M-AG-LAIL-LPVAQYPTIAPPAV--SA-YPGADA-TVQDTV

AcrE:    62 TQVIEQNMNGIDNLMYMSSnSDStGtVqITLTFeSGTDaDIAQVQVQNKLQLAmPLLPQEV
EnvD:    62 TQVIEQNMNGIDNLMYMSStSDSaGsVtITLTFqSGTDpDIAQVQVQNKLQLAtPLLPQEV
consensus   TQVIEQNMNGIDNLMYMSS-SDS-G-V-ITLTF-SGTD-DIAQVQVQNKLQLA-PLLPQEV

AcrE:   123 QQQGvSVEKSSSSfLMVvGvintdgtmTQeDISDYVAaNmKDaiSRtsGVGDVQLFGsQYA
EnvD:   123 QQQGiSVEKSSSSyLMVaGfvsdnpgtTQdDISDYVAsNvKDtlSRlnGVGDVQLFGaQYA
consensus   QQQG-SVEKSSSS-LMV-G--------TQ-DISDYVA-N-KD--SR--GVGDVQLFG-QYA

AcrE:   184 MRIWmnpneLNKfqLTPVDVItaiKaQNaQvAAGQLGGTppvkGQQLNASIIAQTRltstE
EnvD:   184 MRIWldadlLNKykLTPVDVInqlKvQNdQiAAGQLGGTralpGQQLNASIIAQTRfknpE
consensus   MRIW-----LNK--LTPVDVI---K-QN-Q-AAGQLGGT----GQQLNASIIAQTR----E

AcrE:   245 EFGKilLkVNqDGSrVlLrDVAkiELGGENYdiIAefNGqPasGLGIKLATGANALDTAaA
EnvD:   245 EFGKvtLrVNsDGSvVrLkDVArvELGGENYnvIAriNGtPppGLGIKLATGANALDTAkA
consensus   EFGK--L-VN-DGS-V-L-DVA--ELGGENY--IA--NG-P--GLGIKLATGANALDTA-A

AcrE:   306 IrAeLAkmePFFPsGlKivYPYDTTPFVkiSIHEVVKTLvEAIiLVFLVMYLFLQNfRATL
EnvD:   306 IkAkLAelqPFFPqGmKvlYPYDTTPFVqlSIHEVVKTLfEAImLVFLVMYLFLQNmRATL
consensus   I-A-LA---PFFP-G-K--YPYDTTPFV--SIHEVVKTL-EAI-LVFLVMYLFLQN-RATL

AcrE:   367 IPTIAVPVVLLGTFAvLAAFGfSINTLTMFGMVLAIGLLVDDAIVVVENVERVMaEegLPP
EnvD:   367 IPTIAVPVVLLGTFAiLAAFGySINTLTMFGMVLAIGLLVDDAIVVVENVERVMmEdkLPP
consensus   IPTIAVPVVLLGTFA-LAAFG-SINTLTMFGMVLAIGLLVDDAIVVVENVERVM-E--LPP

AcrE:   428 KEATrKSMgQIQGALVGIAMVLSAVFvPMAFFGGSTGAIYRQFSITIVSAMALSVLVALIL
EnvD:   428 KEATeKSMsQIQGALVGIAMVLSAVFiPMAFFGGSTGAIYRQFSITIVSAMALSVLVALIL
consensus   KEAT-KSM-QIQGALVGIAMVLSAVF-PMAFFGGSTGAIYRQFSITIVSAMALSVLVALIL
```

FIG. 5—*Continued.*

bacterial outer membranes show reasonable permeability to hydrophobic molecules. Second, if the main difference between the *acrA* mutant and the wild type was the outer membrane permeability, one would expect that the rates of accumulation of acriflavine would be different in the absence of pumps. Figure 6 shows that there was no detectable difference between the accumulation rates after the addition of CCCP. (iii) This difference could be the consequence of the formation of channels for drugs in the mutant as a result of the loss of structural integrity of AcrAE transporter. In this situation, the drug hypersusceptibilities observed in N43 would be the indirect effect caused by IS*2* insertion. (iv) This

```
    AcrE:  489 TPALCATmLKPiakgdHgEgKkGFFGWFNrmFekSthHYTdSVGgILrSTGRYLvlYliIV
               ||||||| |||     | | | ||||||| | | ||| ||| || |||||| | ||
    EnvD:  489 TPALCATlLKP vsaeHhEnKgGFFGWFNttFdhSvnHYTnSVGkILgSTGRYLliYalIV

consensus      TPALCAT-LKPi----H-E-K-GFFGWFN--F--S--HYT-SVG-IL-STGRYL--Y--IV


    AcrE:  550 vGMayLFvRLPSSFLPdEDQGVFmTMvQLPAGATQERTQKVLneVThYYLtkEKnNVESVF
                ||  || ||||||||| |||||| || |||||||||||||| || |||  || ||||||
    EnvD:  549 aGMvvLFlRLPSSFLPeEDQGVFlTMiQLPAGATQERTQKVLdqVTdYYLknEKaNVESVF

consensus      -GM--LF-RLPSSFLP-EDQGVF-TM-QLPAGATQERTQKVL--VT-YYL--EK-NVESVF


    AcrE:  611 aVNGFgFaGrgQNtGiAFVSLKdWadRpGeENkvEAitmRAtrafsqIkDamVfaFNlPAI
                |||| | |  || | |||||| |  | | ||  ||   ||       | |  |  || |||
    EnvD:  610 tVNGFsFsGqaQNaGmAFVSLKpWeeRnGdENsaEAvihRAkmelqkIrDqfVipFNmPAI

consensus      -VNGF-F-G--QN-G-AFVSLK-W--R-G-EN--EA---RA------I-D--V--FN-PAI


    AcrE:  672 VELGTATGFDFELIDQAGLGHekLTQARNQLLaeAAkHPdmLtSVRPNGLEDTpQFKidiD
               |||||||||||||||||||||  |||||||||  || ||  | ||||||||| |||   |
    EnvD:  671 VELGTATGFDFELIDQAGLGHdaLTQARNQLLgmAAqHPasLvSVRPNGLEDTaQFKlevD

consensus      VELGTATGFDFELIDQAGLGH--LTQARNQLL--AA-HP--L-SVRPNGLEDT-QFK---D


    AcrE:  733 QEKAQALGVSinDINtTlgaAwGGsYVNDFIDRGRVKKvYVmseAKyRMLPdDigdwYVRa
               ||||||||||  ||| |   | || |||||||||||||| ||   || |||| |    |||
    EnvD:  732 QEKAQALGVSlsDINqTistAlGGtYVNDFIDRGRVKKlYVqadAKfRMLPeDvdklYVRs

consensus      QEKAQALGVS--DIN-T---A-GG-YVNDFIDRGRVKK-YV---AK-RMLP-D----YVR-


    AcrE:  794 AdGqMVPFSAFssSrWeYGSPRLERYNGLPSMEIlGqAAPGkStgeAMeLMEqLASKLPtG
               | | |||||||  | | |||||||||||||||| | |||| |   || ||| |||||| |
    EnvD:  793 AnGeMVPFSAFttShWvYGSPRLERYNGLPSMEIqGeAAPGtSsadAMaLMEnLASKLPaG

consensus      A-G-MVPFSAF--S-W-YGSPRLERYNGLPSMEI-G-AAPG-S---AM-LME-LASKLP-G


    AcrE:  855 vGYDWTGMSYQERLSGNQAPsLyAISliVVFLCLAALYESWSIPfSVMLVVPLGviGaLLA
                |||||||||||||||||| | |||  |||||||||||||||| |||||||||  | |||
    EnvD:  854 iGYDWTGMSYQERLSGNQAPaLvAISfvVVFLCLAALYESWSIPvSVMLVVPLGivGvLLA

consensus      -GYDWTGMSYQERLSGNQAP-L-AIS--VVFLCLAALYESWSIP-SVMLVVPLG--G-LLA


    AcrE:  916 ATfrgltNDVYFqVGLLTTIGLSAKNAILIVEFAKDLMdKEGKGliEATLdAVRMRLRPIL
               ||     ||||| |||||||||||||||||||||||| |||||  |||| ||||||||||
    EnvD:  915 ATlfnqkNDVYFmVGLLTTIGLSAKNAILIVEFAKDLMeKEGKGvvEATLmAVRMRLRPIL

consensus      AT-----NDVYF-VGLLTTIGLSAKNAILIVEFAKDLM-KEGKG--EATL-AVRMRLRPIL


    AcrE:  977 MTSLAFILGVmPLvIStGAGSGAQNAVGtGVMGGMVtATvLAIFFVPVFFVVvrRRfsrkn
               |||||||||| || || |||||||||| ||||||| || ||||||||||||  ||
    EnvD:  976 MTSLAFILGVlPLaISnGAGSGAQNAVGiGVMGGMVsATlLAIFFVPVFFVV iRRcfkg.

consensus      MTSLAFILGV-PL-IS-GAGSGAQNAVG-GVMGGMV-AT-LAIFFVPVFFVV -RR----


    AcrE: 1038 ediehshtvdhh.

    EnvD: 1035

consensus
```

FIG. 5—*Continued.*

TABLE 3. Comparison of MICs of various dyes, detergents, and antibiotics for N43 and PM61 and their isogenic wild-type parents, W4573 and P678S^R

Compound	MIC (μg/ml) for:			
	W4573 (*acrA*$^+$)	N43 (*acrA*)	P678S^R (*envC*$^+$)	PM61 (*envC*)
Ethidium bromide	512	16	>512	128
Novobiocin	128	4	512	128
Actinomycin D	256	256	256	4
Vancomycin	256	256	256	4
Penicillin G	32	16	64	4
Erythromycin	256	8	256	4
Fusidic acid	256	8	256	16
Mitomycin C	8	0.25	16	0.125
Sodium dodecyl sulfate	>10,000	64	>10,000	128
Acriflavin	>64[a]	32	>64	32
Crystal violet	16	1	64	4
Rifampin	16	8	32	4
Ampicillin	4	2	4	1
Tetracycline	2	0.25	1	0.125

[a] Acriflavine concentrations greater than 64 μg/ml were not tested because of the drug's limited solubility in LB medium.

TABLE 4. Effects of AcrA dosage (mediated by plasmid copy number) and the existence of AcrE on the drug susceptibilities of N43

Compound	MIC (μg/ml) for:				
	W4573	N43	N43 + pLA[a]	N43 + pHA[b]	N43 + pUC151A[c]
Ethidium bromide	512	16	512	512	>512
Novobiocin	128	4	64	128	256

[a] Derived from the low-copy-number vector pACYC177; contains the insertion sequence which encodes the intact AcrA and the N-terminal 40 amino acid residues of AcrE.

[b] Derived from the high-copy-number vector pUC19; contains the insertion sequence which encodes the intact AcrA and the N-terminal 101 amino acid residues of AcrE.

[c] Derived from the high-copy-number vector pUC19; contains the insertion sequence which encodes the intact AcrA and AcrE.

difference could be caused by the AcrAE proteins pumping out acriflavine as an efflux pump. Some support for this model comes from the homology between AcrE and CzcA (26% amino acid identity; data not shown). CzcA is a plasmid-encoded, 1,063-residue polypeptide which has been suggested to promote divalent cation efflux in *Alcaligenes eutrophus* (34). In this model, however, the AcrAE system cannot be the only effective pump for acriflavine. This is clear from the observation that even *acrA* cells can extrude much acriflavine (compare the pre-CCCP and post-CCCP levels of acriflavine in Fig. 6). The homology among AcrAE, EnvCD, and the carboxyl terminus of a putative ORF, which has been identified and located around 53 min of *E. coli* genetic map by Bouvier et al. (2), suggests that they may represent a new family of proteins with similar functions. Therefore, there may be multiple pumps in *E. coli* designed in such a way that they have not only different but also overlapping substrates to allow the optimum control of both specificities and capacities. We are currently designing experiments to test models iii and iv.

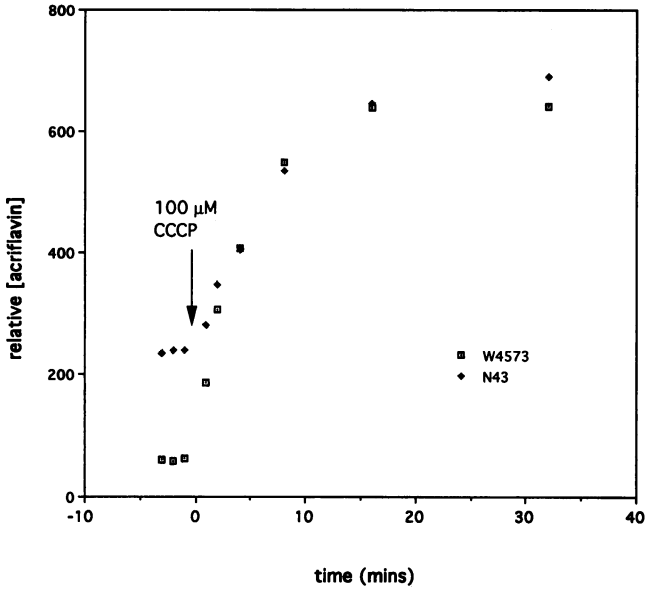

FIG. 6. Energy-dependent acriflavine uptake in W4573 and N43. Cells were grown in LB containing 2.5 μg of acriflavine per ml. At time zero, 100 μM CCCP was added to stop the pump-out process. The relative drug concentrations at various time points within the two strains were determined by the fluorescence measurements. No difference in the accumulation of acriflavine after the addition of CCCP can be seen between W4573 and N43.

Despite the observations that some mutations at the *acrAE* or *envCD* loci increase drug susceptibilities in *E. coli*, the exact functions of these genes remain unclear. Both N43 and PM61 display pleiotropic phenotypes even in the absence of drugs, and this finding indicates more general roles for these polypeptides in *E. coli*. In addition to the drug-susceptible phenotype, N43 is susceptible to high concentrations of sodium ion in the growth medium (32; our unpublished data). The *acrA* allele has also been shown to interact with the *adk* gene (formerly *plsA*, encoding adenylate kinase) (31). Mutations at the *acrA* locus seem to enable Δ*topA* mutants to survive under specific growth conditions of low osmolarity (6). On the other hand, the *envCD* mutation in PM61 causes susceptibility to phospholipase C (41) and filamentous growth at high temperatures (16). Some morphological change of the inner membrane was reported in N43 in the presence of acriflavine (30, 33). A change in phospholipid composition was also observed in PM61 (25).

How mutations at the *acrAE* locus help Δ*topA* strains survive is an intriguing question. Liu and Wang have shown that transcription can generate supercoiling (20) because the topology of transcription requires a relative rotation between RNA polymerase and DNA (7). Using plasmids as a model system, we have shown that the generation of transcription-induced supercoiling is very inefficient during transcription of cytosolic genes (4). On the other hand, transcription of membrane genes, particularly those encoding integral inner membrane proteins like *tet*, can lead to the rapid accumulation of negative supercoils on the DNA template under Δ*topA* background (4; our unpublished data; also see reference 21). This is probably due to the membrane anchoring of RNA polymerase through the coupling of transcription, translation, and membrane insertion of the nascent polypeptides. Presumably, expression of some chromosomal genes encoding integral membrane proteins is deleterious in Δ*topA* strains because of the high level of localized negative supercoiling which could result.

One potential mechanism to help Δ*topA* strains would be that mutation at the *acrAE* loci relieves the transcription-induced superhelical stress by decreasing the coupling efficiency between transcription, translation, and membrane insertion. It has been observed that an *envCD* mutant displays a phenomenon known as delayed tetracycline resistance (14a). It will be interesting to determine whether delayed expression of tetracycline resistance in PM61 is caused by the inefficient membrane insertion of the *tet* gene product. Other alternative possibilities would be that mutation at the *acrAE* locus leads to a direct change of chromosomal structure or a lower than normal gyrase activity in vivo.

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ADDENDUM

After the completion of this work, we became aware that sequencing of the same genes was reported recently by Xu et al. (43).

ADDENDUM IN PROOF

Seiffer et al. have recently shown that EnvC is indeed a lipoprotein of the cytoplasmic membrane of *E. coli* (D. Seiffer, J. R. Klein, and R. Plapp, FEMS Microbiol. Lett. 107:175–178, 1993).

REFERENCES

1. **Bachmann, B. J.** 1990. Linkage map of *Escherichia coli* K-12, edition 8. Microbiol. Rev. **54:**130–197.
2. **Bouvier, J., C. Richaud, W. Higgins, O. Bogler, and P. Stragier.** 1992. Cloning, characterization, and expression of the *dapE* gene of *Escherichia coli*. J. Bacteriol. **174:**5265–5271.
3. **Coleman, W. G., and L. Leive.** 1979. Two mutations which affect the barrier function of the *Escherichia coli* K-12 outer membrane. J. Bacteriol. **139:**899–910.
4. **Cook, D. N., D. Ma, N. G. Pon, and J. E. Hearst.** 1992. Dynamics of DNA supercoiling by transcription in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **89:**10603–10607.
5. **DiNardo, S., K. A. Voelkel, and R. Sternglanz.** 1982. *Escherichia coli* DNA topoisomerase I mutants have compensatory mutations in DNA gyrase genes. Cell **31:**43–51.
6. **Dorman, C. J., A. S. Lynch, N. Ni Bhriain, and C. F. Higgins.** 1989. DNA supercoiling in *Escherichia coli*: topA mutations can be suppressed by DNA amplifications involving the tolC locus. Mol. Microbiol. **3:**531–540.
7. **Gamper, H. B., and J. E. Hearst.** 1982. A topological model for transcription based on unwinding angle analysis of *E. coli* RNA polymerase binary, initiation, and ternary complexes. Cell **29:**81–90.
8. **Gellert, M., K. Mizuuchi, M. H. O'Dea, and H. A. Nash.** 1976. An enzyme that introduces superhelical turns into DNA. Proc. Natl. Acad. Sci. USA **73:**3872–3876.
9. **Ghosal, D., and H. Saedler.** 1979. Nucleotide sequence of the transposable DNA-element IS2. Nucleic Acids Res. **6:**1111–1122.
10. **Hayashi, S., and H. C. Wu.** 1990. Lipoproteins in bacteria. J. Bioenerg. Biomembr. **22:**451–471.
11. **Higgins, C. F.** 1992. ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. **8:**67–113.
12. **Jacob, F., and E. L. Wollman.** 1961. Sexuality and the genetics of bacteria, p. 60. Academic Press, New York.
13. **Jaurin, B., and S. Normark.** 1983. Insertion of IS2 creates a novel ampC promoter in *Escherichia coli*. Cell **32:**809–816.
14. **Kirkegaard, K., and J. C. Wang.** 1985. Bacterial DNA topoisomerase I can relax positively supercoiled DNA containing a single-stranded loop. J. Mol. Biol. **185:**625–637.

14a. **Klein, J. R., B. Henrich, and R. Plapp.** 1990. Molecular cloning of the *envC* gene of *Escherichia coli*. Curr. Microbiol. **21:**341–347.

15. **Klein, J. R., B. Henrich, and R. Plapp.** 1991. Molecular analysis and nucleotide sequence of the envCD operon of *Escherichia coli*. Mol. Gen. Genet. **230:**230–240.
16. **Klein, J. R., and R. Plapp.** 1992. Locations of the *envCD* genes on the physical map of the *Escherichia coli* chromosome. J. Bacteriol. **174:**3828–3829.
17. **Kohara, Y., K. Akiyama, and K. Isono.** 1987. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell **50:**495–508.

17a. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.

18. **Leive, L., S. Telesetsky, W. G. Coleman, and D. Carr.** 1984. Tetracyclines of various hydrophobicities as a probe for permeability of *Escherichia coli* outer membranes. Antimicrob. Agents Chemother. **25:**539–544.
19. **Levy, S.** 1992. Active efflux mechanisms for antimicrobial resistance. Antimicrob. Agents Chemother. **36:**695–703.
20. **Liu, L. F., and J. C. Wang.** 1987. Supercoiling of the DNA template during transcription. Proc. Natl. Acad. Sci. USA **84:**7024–7027.
21. **Lynch, A. S., and J. C. Wang.** 1993. Anchoring of DNA to the bacterial cytoplasmic membrane through cotranscriptional synthesis of polypeptides encoding membrane proteins or proteins for export: a mechanism of plasmid hypernegative supercoiling in mutants deficient in DNA topoisomerase I. J. Bacteriol. **175:**1645–1655.
22. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82:**8129–8133.
23. **Manoil, C., J. J. Mekalanos, and J. Beckwith.** 1990. Alkaline phosphatase fusions: sensors of subcellular location. J. Bacteriol. **172:**515–518.
24. **Menzel, R., and M. Gellert.** 1983. Regulation of the genes for *E. coli* DNA gyrase: homeostatic control of DNA supercoiling. Cell **34:**105–113.
25. **Michel, G., D. DiSavino, and J. Starka.** 1977. Phospholipid composition and phenotypic correction of an *envC* division mutant of *Escherichia coli*. J. Bacteriol. **129:**145–150.
26. **Miller, J. H.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Nakamura, H.** 1965. Gene-controlled resistance to acriflavin and other basic dyes in *Escherichia coli*. J. Bacteriol. **90:**8–14.
28. **Nakamura, H.** 1968. Genetic determination of resistance to acriflavin, phenethyl alcohol, and sodium dodecyl sulfate in *Escherichia coli*. J. Bacteriol. **96:**987–996.
29. **Nakamura, H.** 1974. Plasmid instability in acrA mutants of *Escherichia coli* K12. J. Gen. Microbiol. **89:**85–93.
30. **Nakamura, H., and A. Suganuma.** 1972. Membrane mutation associated with sensitivity to acriflavin in *Escherichia coli*. J. Bacteriol. **110:**329–335.
31. **Nakamura, H., T. Tojo, and J. Greenberg.** 1975. Interaction of the expression of two membrane genes, *acrA* and *plsA*, in *Escherichia coli* K-12. J. Bacteriol. **122:**874–879.
32. **Nakamura, H., H. Yamamoto, K. Hirase, and A. Hase.** 1990. A role for the acrA gene in the protection of *Escherichia coli* cells against excess sodium. Plant Cell Physiol. **31:**649–654.
33. **Nakamura, H., E. Yokomura, A. Hase, and A. Suganuma.** 1981. A plasma membrane mutation acrA in *Escherichia coli*. Plant

Cell Physiol. **23**:1141–1148.
34. **Nies, D., A. Nies, L. Chu, and S. Silver.** 1989. Expression and nucleotide sequence of a plasmid-determined divalent cation efflux system from Alcaligenes eutrophus. Proc. Natl. Acad. Sci. USA **86**:7351–7355.
35. **Nikaido, H., and M. Vaara.** 1985. Molecular basis of bacterial outer membrane permeability. Microb. Rev. **49**:1–32.
36. **Plesiat, P., and H. Nikaido.** 1992. Outer membranes of Gram-negative bacteria are permeable to steroid probes. Mol. Microbiol. **6**:1323–1333.
37. **Pruss, G. J., S. H. Manes, and K. Drlica.** 1982. Escherichia coli DNA topoisomerase I mutants: increased supercoiling is corrected by mutations near gyrase genes. Cell **31**:35–42.
38. **Raji, A., D. J. Zabel, C. S. Laufer, and R. E. Depew.** 1985. Genetic analysis of mutations that compensate for loss of *Escherichia coli* DNA topoisomerase I. J. Bacteriol. **162**:1173–1179.
39. **Rudd, K. E., W. Miller, J. Ostell, and D. A. Benson.** 1990. Alignment of *Escherichia coli* K12 DNA sequences to a genomic restriction map. Nucleic Acids Res. **18**:313–321.
40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
41. **Starkova, Z., N. Bonnaverio, and J. Starka.** 1981. Hydrolysis of phospholipids by phospholipase C in intact cells of wild-type and envelope mutants of *Escherichia coli* K12. FEBS Lett. **130**:261–264.
42. **Stein, W. C.** 1967. The movement of molecules across the cell membranes. Academic Press, New York.
43. **Xu, J., M. L. Nilles, and K. P. Bertrand.** 1993. Abstr. 93rd Gen. Meet. Am. Soc. Microbiol. 1993, abstr. K-169, p. 290.
44. **Yamaguchi, K., F. Yu, and M. Inouye.** 1988. A single amino acid determinant of the membrane localization of lipoproteins in *E. coli*. Cell **53**:423–432.